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Scutellaria Biotechnology: Achievements and Future Prospects

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Abstract. Plants of the genus *Scutellaria* (Family Lamiaceae) constitute one of the common components of Eastern as well as traditional American medicine. Skullcap is a North American perennial plant belonging to the genus *Scutellaria*. The genus is widespread in Northern Hemisphere represented by close to 400 species. Many species are rare, threatened, or endangered. Habitat destruction, urbanization, and poor seed set are the few reasons behind diminishing population of many skullcaps. Many skullcaps have showy, beautiful blooms with great potential as ornamental plants. Skullcap are used in alternative medicine as anti-inflammatory, antispasmodic, emmenagogue, nervine, sedative and strong tonic. We have developed a germplasm collection at Fort Valley State University and the populations are maintained in the greenhouse and through micropropagation. We have made significant headway in the areas of micropropagation, transformation for desired gene transfer and hairy root induction, extraction and HPLC analysis of targeted flavonoids, and clinical role of select flavonoids using glioma cell lines.

Keywords: Micropropagation, conservation, medicinal plant, hairy root culture, anti-tumor, flavonoids

INTRODUCTION

Recent trends in medicinal plants research show that while ethnobotanical surveys continue, there is an appreciable increase in research activity in the area of bioactivity of natural products. As many as 84% of pediatric oncology patients, 50% of breast cancer patients and 37% of prostate cancer patients use complementary and alternative medicine (CAM), including predominantly herbal approaches (Richardson, 2001).

Scutellaria is a perennial, herbaceous genus in the Lamiaceae family with 350 - 400 species (Paton, 1990; Cole *et al*., 2007). This genus is well represented by about 90 species in the North America. The scullcap (skullcap) is a North American perennial plant that grows in wet places in Canada and the northern and eastern U.S.

Medicinal plants are gathered from the wild depleting natural resources at an alarming rate. The challenge is to produce plants economically and market their products as demanded by the industry and consumers. Though, many pharmaceutical and nutraceutical companies market products based on traditional medicine and scientific research, serious problems may arise with the adaptation of these traditional medicines. Misidentification of a medicinal plant resulted in the loss of renal function through irreversible interstitial fibrosis in more than 100 patients (Betz, 1998).

Successful micropropagation of many *Scutellaria* species have been reported (Li *et al*., 2000; Sinha *et al*., 1999; Joshee *et al*., 2002, 2007; Tascan, 2007; Tascan *et al*., 2007, 2009, 2010, and Stojakowska *et al*., 1999). It has been estimated that the annual consumption of scullcap increased by 23% between 2000 and 2001. The dollar value of the 2001 scullcap harvest was between \$185,000 and \$195,000, that was 3.5 times higher than that of 1997 (Greenfeild and Davis, 2004). As the demand for medicinal herb is increasing, there is renewed interest in this genus to propagate clean plants and biomass in large amount (Tascan, 2007; Tascan *et al.*, 2010).

Scullcap is currently recommended as an alternative sedative treatment to kava which could increase the demand for this herb. The price of scullcap has steadily increased during the past five years and is currently around \$8.80-17.60/kg. In Canada organically grown scullcaps fetch premium price of \$17.60-33.00/kg (Porter, 2006). The demand for scullcap in the world market is predicted to grow at an annual rate of 20-30% (Greenfeild and Davis, 2004). During 2001, about 70% of the world market demand for scullcap was supplied by sources outside of N. America.

S. baicalensis is the most extensively studied species of Skullcap and its root is known to contain a number of flavone derivatives. A metabolomic analysis of *Scutellaria baicalensis* shows that, plant contains more than 2000 compound and 781 are putatively medicinal (Murch *et al*., 2004). The chemical constituents have also been investigated in other *Scutellaria* species including *S. rivularis* Wall. (Chou, 1978; Tomimori *et al*., 1984, 1986a, 1990), *S. discolor* (Tomimori *et al*., 1985a, 1986b), *S. indica* (Chou and Lee, 1986; Miyaichi *et al*., 1987, 1989) and *S. scandens* (Miyaichi *et al*., 1988a, b).

The possible use of plant cell cultures for the specific biotransformation of natural compounds has been demonstrated (Berlin, 1988; Verpoorte *et al*., 2000; Vanisree *et al*., 2004: Sarin, 2005). Production of flavonoids has been reported from tissue cultures of a number of plant species such as *Cicer arietinum*, *Crotolaria juncea*, *Emblica officinalis*, and *Tylophora indica* (Sarin, 2005). Organ and callus culture techniques have been used to study phenolic compound biosynthesis in *Scutellaria*. Using the hairy-root culture system, Hirotani (1999), isolated a new flavone glucoside, 15 known flavonoids, and 5 known phenylethanoids. Transformed root culture of *S. baicalensis* induced by *A. rhizogenes* (ATCC 15834) produced both aglucuronide type flavonoids (baicalein, wogonin) and glucuronide type flavanoids (baicalin). Flavonoid amount (glucuronide and aglucuronide flavonoids) in root portion differed among regenerated plantlets. Hairy root culture induced using LBA 9402 strain of *A. rhizogenes* yielded 6.87±0.49% baicalin and 1.51±0.06% wogonin on dry weight basis (Stojakowska and Malarz, 2000).

Natural plant products have been invaluable as tools for deciphering the logic of biosynthesis and as platforms for developing frontline drugs (Newman *et al*., 2000). Scientific studies on the extracts or isolated active components from *Scutellaria* have been mostly limited to their anti-inflammatory activities (Nijveldt *et al*., 2001; Huang *et al*., 2006). Baicalin inhibits superantigen-induced inflammatory cytokines and chemokines (Krakauer *et al*., 2001). *Scutellaria baicalensis* and some of its constituents are also suggested to enhance TGF-β1 gene expression in RAW 264.7 murine macrophage cell line (Chuang *et al*., 2005).

MATERIALS AND METHODS

We have been working on the conservation, reproductive biology, micropropagation, genetic transformation to modify/introduce new traits using *A. tumefaciens* and induction of hairy root cultures to study secondary metabolite biosynthesis (Sinha *et al*., 1999; Joshee *et *al*., 2002, 2007; Parajuli *et al*., 2009, 2010; Tascan, 2007; Tascan *et al*., 2007, 2009, 2010; Wu *et al*., 2009). All scanning electron microscopic work was conducted at the Ctr. for Ultrastructural Research, University of Georgia, Athens, USA. We have continuously enriched our germplasm collection and at present have 15 species maintained as tissue cultures and potted plants in the greenhouse. We are also studying phytochemical profile of these species and the activity of total extracts and its constituents on various cancer cell lines and animal model (Parajuli *et al*., 2009, 2010). Most of the methods used are standard in our lab and have been described in detail in our publications cited in this section.

RESULTS AND DISCUSSION

Scutellaria species have a great potential as ornamental plants for floriculture industry. Flowers are bright (Fig 1A, B, and C) and blooming period in up to three months. A large variability exists in the size and structures of floral appendages. Anthers lobes contain hair to trap pollen grains upon dehiscence and release them in small groups to insure pollination (Fig. 1. D). Fig. 1E is the scanning electron micrograph of the stigma surface in *S. ocmulgee*.

Micropropagation: We have conducted micropropagation studies in many species of *Scutellaria* and most of the species respond pretty well in the MS and/or B5 media (Murashige and Skoog, 1962; and Gamborg *et al*., 1968). Most responsive explants are shoot tips and nodal segments (Sinha *et al*., 1999; Joshee *et al*., 2007). In general benzyl adenine (1-5µM) is the best cytokinin for shoot bud induction and these buds can be easily elongated in a basal medium. These elongated shoots or microcuttings can be successfully rooted in MS medium with 5µM indole butyric acid (IBA). Most microcutting strike roots within one to two weeks. These rooted plantlets can be transitioned to outside environment by gradually reducing humidity and increasing light intensity (Fig. 1. I). We have studies the effect of liquid culture in the multiplication stage by transferring explants with shoot buds into Liquid Lab Rocker system. Some of the species (*S. barbata*) do very well in the liquid medium (Fig. 1.H) whereas other turn hyperhydric to various degree (Tascan *et al*., 2010). Hyperhydricity basically depends on sucrose concentration, cytokinin used and the type of agar (Ziv, 1995; Gollagunta *et al*., 2005). Hyperhydricity causes reduced multiplication rate, poor quality shoot and death of cell and tissues. Inserting a filter paper as a substratum has helped in many cases.

Genetic transformation studies on Scutellaria: We are interested in using *Scutellaria* genus as a model system to understand flavonoids biosynthesis in plants and hence initiated genetic transformation studies using *Agrobacterium tumefaciens* and *A. rhizogenes* both. We have initiated transformation studies on *S. ocmulgee* as it responds well in tissue culture and also proved useful in inducing apoptosis and necrosis in tumor cells (Parajuli *et al*., 2009, 2010). Hairy root cultures were initiated on American skullcap *S. lateriflora* as it is already sold commercially as a medicinal herb.

S. ocmulgee was transformed via *Agrobacterium tumefaciens* strain EHA105 harboring plasmid pq35SGR following the method of Li *et al*., 2004. The *Agrobacterium* culture was grown overnight in 20 mL YEP medium containing 20 mg L^{-1} rifampicin and 50 mg L^{-1} kanamycin on a rotary shaker (200 rpm) at 28°C. The pq35GR vector consists of the cauliflower mosaic virus (CaMV) 35S promoter-derived bi-directional promoters containing two divergently arranged enhancer repeats, a fusion between the nptII and GUS genes, and the EGFP gene. After co-cultivation with *A. tumefaciens* in the dark at 28°C for 3 days, the explants were rinsed once with 500 mg/L carbenicillin for 5 min and subsequently placed on MS medium supplemented with 300 mg/L carbenicillin and 50 mg/L kanamycin. Explants were subcultured every two weeks and transformed plants exhibited high expression of green fluorescent protein (Fig. 1. J).

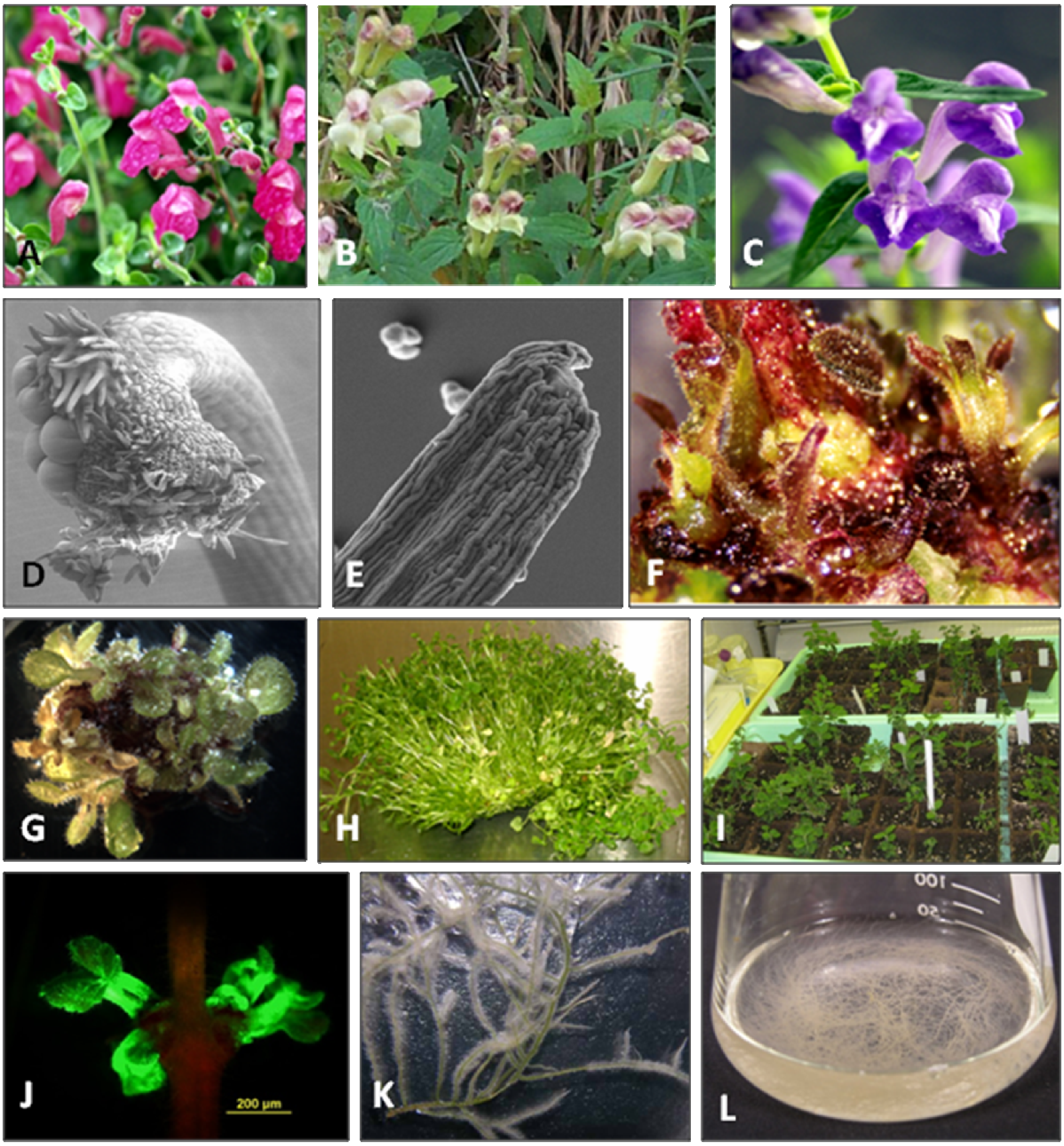
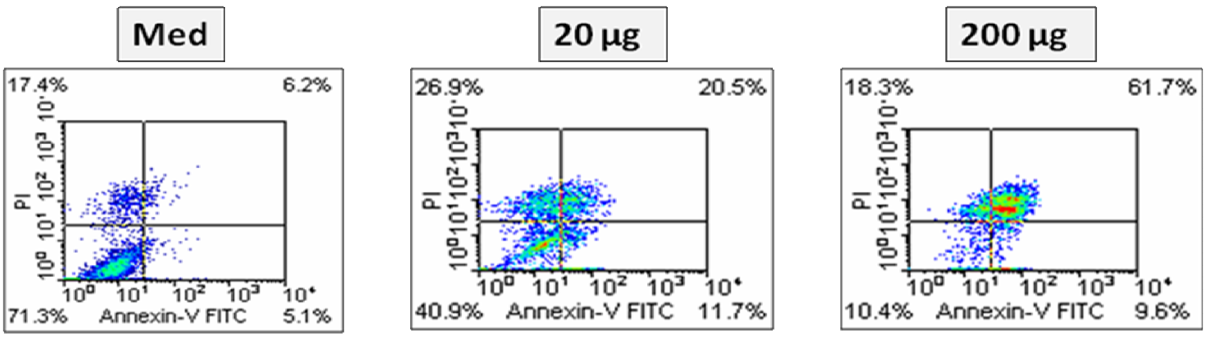


Fig. 1. Various aspects of tissue culture research A. *Scutellaria suffrutescens*, B. *S. angulosa*, C. *S. baicalensis*, D. Scanning electron micrograph (SEM) of a *S. lateriflora* stamen showing hair capturing pollen grains for controlled release (165X), E. SEM of the receptive surface of stigma in *S. ocmulgee* (165 X), F. Multiple shoot induction in the endangered *S. montana* using shoot tip as an explant, G. Multiple shoot induction in rare *S. ocmulgee* using nodal explant, H. Very high multiplication rate was achieved using liquid culture system in *S. barbata*, I. Elongated shoots are hardened and processed for potting in the greenhouse, J. *Agrobacterium tumefaciens* mediated genetic transformation exhibiting GFP expression, K and L. *Agrobacterium rhizogenes* (strain 15834) mediated hairy root culture initiation and their continuous growth in a flask.

Hairy roots were induced by infecting stem *S. lateriflora* explants with *Agrobacterium rhizogenes* strain ATCC 15834 (Wu *et al*., 2009; Fig. 1. K). Molecular characterization of the hairy roots was done by PCR targeting the transferred *rol*C and *aux*2 genes from *A. rhizogenes*. PCR of the *vir*D2 gene was used to discard any remaining agrobacteria in the root tissue. Several lines of *S. lateriflora* hairy roots were selected based on their growth performance in solid and liquid media (Fig. 1 L). In order to study the production of the bioactive flavonoids, the hairy roots are being treated with different elicitors and the chemical profiles will be studied by high performance TLC and HPLC analyses.

Anti-tumor activities: Studies in our lab have demonstrated tumor-specific and dose-dependent anti-proliferative and pro-apoptotic activities of leaf, stem and root extracts obtained from various *Scutellaria* species, including *S. angulosa*, *S. baicalensis*, *S. integrifolia*, *S. ocmulgee*, *S. montana*, *S. scandens* and *S. suffrutescens* against malignant breast cancer, glioma and prostate cancer cells *in vitro* as well as *in vivo* (Parajuli *et al.*, 2009). An example of the apoptosis induction in U87-MG glioma cells using leaf extracts of *S. integrifolia* has been shown in Figure 2. Further, comparisons of the cellular effects induced by the entire extract versus the four-compound combination produced comparable cell cycle changes, and levels of growth inhibition. Individual compounds exhibited antiandrogenic activities with reduced expression of the androgen receptor and androgen-regulated genes.

We have compared the anti-tumor activities of six predominant *Scutellaria* flavonoids, namely, apigenin, baicalein, baicalin, chrysin, scutellarein and wogonin against breast cancer, glioma and prostate cancer cells *in vitro.* All these flavonoids significantly inhibited the proliferation, induced G1/G2 arrest and enhanced apoptosis in the three cell lines tested (Parajuli *et al*., 2009). Similar to the results of *Scutellaria* extracts, as mentioned above, anti-tumor activities of all six flavonoids also involved significant inhibition of Akt / PKB signaling activity in the tumor cells (Parajuli *et al.*, 2010). Similar results have been seen in the trials involving medicine BZL 101 (*S. barbata*) that inhibits breast cancer cell lines by inducing apoptosis. In a phase I clinical trial, BZL101 was safe and had a favorable toxicity profile (Rugo *et al*., 2007). *Scutellaria barbata* extract has also been shown to be effective in a different cancer cell line (human promyelocytic leukemia HL-60) inducing inhibition of growth and a G_1 phase arrest of the cell cycle in a concentration- and time-dependent manner (Kim *et al*.,2007).



Relative percentage of:	Apoptotic cells	Necrotic cells
Medium (Control)	11.3%	17.4%
Extract 20 mg/mL	32.2%	26.9%
Extract 200 mg/mL	71.3%	18.3%

Fig. 2. Induction of apoptosis in U87-MG glioma cells at 72 hours following treatment with leaf extracts of *S. integrifolia.*

CONCLUSIONS

Demand for a wide variety of wild species is increasing with growth in human needs, numbers and commercial trade. With the increased realization that some wild species are being over-exploited, a number of agencies are recommending that wild species be brought into cultivation systems. Sustainable harvesting needs to be recognized as the most important conservation strategy for most wild-harvested species and their habitats, given their current and potential contributions to local economies and their greater value to harvesters over the long term. It is necessary to understand reproductive biology of these plants in relation to their flowering behavior, pollinators, pollination and fertilization process and seed set. We have seen poor seed set in rare and endangered species and hence their preservation is of immense importance. We have initiated research in the area of low temperature preservation of two rare *Scutellaria* species.

Flavonoids are low molecular weight compounds and their biosynthesis is among the best described secondary metabolic pathways, and in many plants, genes encoding flavonoids biosynthetic enzymes have been cloned and characterized. Our transformation models will help understand the possible role of transcription factors (e.g. MYB) in the expression of a subset of genes in flavonoids biosynthesis pathway. Research on hairy root cultures is aimed at fine tuning of the factors in controlled conditions to produce desired compound preferentially. Development of newer technologies using molecular approach will help making herbal industry safe. With an initial focus on tracking gene expression changes for target identification, microarray applications will assist the entire drug discovery enormously (Gerhold *et al*., 2002; Koppal, 2004). Genomic fingerprinting can differentiate between individuals, species and populations and is useful for the detection of the homogeneity of the samples and presence of adulterants (Sucher and Carles, 2008).

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